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Endophilin and Synaptojanin Hook Up to Promote Synaptic Vesicle Endocytosis

Clathrin-mediated endocytosis of synaptic vesicles requires molecular rearrangements of proteins as well as lipids. In this issue of *Neuron*, Schuske et al. and Verstreken et al. show that the lipid-modifying enzyme endophilin recruits and stabilizes the polyphosphoinositide phosphatase synaptojanin at nerve terminals. This remarkable pairing of two enzymatic activities promotes multiple steps of clathrin-mediated endocytosis of synaptic vesicles.

To sustain neurotransmitter release during periods of high synaptic activity, synaptic vesicle membranes and proteins must be rapidly recaptured and locally recycled. Two kinetically distinct modes may support vesicle recycling (Slepnev and De Camilli, 2000). One is clathrin-mediated endocytosis via coated pits, which occurs at specialized endocytotic zones that are distinct from active zones and recaptures the vesicle membrane after it fully collapses into the plasma membrane after vesicle fusion. The other mode of vesicle retrieval, commonly termed "kiss-and-run," is assumed to recapture the vesicle membrane shortly after the formation of a fusion pore but before the vesicle membrane collapses into the plasma membrane.

The best-characterized mode of synaptic vesicle recycling is clathrin-mediated endocytosis, which can be superficially divided into four major steps (Slepnev and De Camilli, 2000): clathrin coat recruitment, vesicle budding, vesicle fission, and vesicle uncoating. The key players are clathrin and proteins of the adaptor complex forming the coat. Although clathrin coats alone can generate icosahedral cages, the formation of a clathrin-coated pit from a vesicle bilayer is inherently more complex and requires molecular rearrangements within the coat and within the lipid bilayer itself.

Phosphatidylinositol-4,5-biphosphate (PI(4,5)P) is a pleiotrophic regulator of clathrin coat assembly and disassembly (Cremona et al., 1999). Accordingly, hydrolysis of PI(4,5)P2 by the polyphosphoinositide phosphatase synaptojanin is thought to play a critical role during multiple steps of endocytosis. Synaptojanin interacts with a variety of proteins of the endocytic machinery such as endophilin, Dap160/intersectin, and dynamin (Slepnev and De Camilli, 2000). Genetic disruption of synaptojanin in mouse and *C. elegans* suggested multiple roles in recycling, including the uncoating and fission of vesicles (Cremona et al., 1999; Harris et al., 2000; Kim et al., 2002). These deduced roles are consistent with synaptojanin's intrinsic enzymatic activity and/or some of its protein interactions. However, the role of synaptojanin's interaction with endophilin remained unclear.

Endophilin was originally identified as a binding partner for synaptojanin (Slepnev and De Camilli, 2000). Since the SH3 domain of endophilin binds tightly the proline-rich domains of dynamin and synaptojanin, endophilin has been suggested to act as an adaptor protein by recruiting each protein to its site of action. Consistently, manipulations of endophilin's SH3 domain caused an accumulation of deeply invaginated pits and clathrin-coated vesicles at the lamprey giant synapse (Slepnev and De Camilli, 2000). However, studies focusing on endophilin's enzymatic lysophospatidic acid acyl transferase (LPAAT) activity led to a second hypothesis of endophilin function.

The LPAAT activity of endophilin converts lysophosphatidic acid and acyl-CoA into phosphatidic acid. Since the cone shape of phosphatidic acid is more compatible with a high curvature index than the inverted cone shape of lysophospatidic acid, this suggested the intriguing possibility that endophilin promotes a negative membrane curvature by altering the composition of the donor membrane at the base of the invaginating bud, thereby promoting the conversion of a shallow into a deeply invaginated pit (Slepnev and De Camilli, 2000). Consistently, clathrin-mediated synaptic vesicle endocytosis is blocked in *Drosophila* endophilin mutants, and shallow invaginated pits in the presynaptic membrane are frequently observed (Guichet et al., 2002; Verstreken et al., 2002). Since LPAAT activity might be dispensable for membrane deformation (Farsad et al., 2001), endophilin's major role in vesicle endocytosis remained controversial.

Coming from opposite sides and using different genetics system, two reports in this issue of *Neuron* shed light onto the significance of the endophilin-synaptojanin interaction. Verstreken et al. and Schuske et al. independently demonstrate that *Drosophila* synaptojanin and *C. elegans* endophilin are critical for clathrin-mediated endocytosis. Intrigued by a remarkable similarity of endocytotic defects caused by loss of endophilin and synaptojanin, both groups go on to convincingly show that endophilin's and synaptojanin's functions are tightly interweaved, if not inseparable.

Hugo Bellen's group (Verstreken et al., 2003) identified two lethal point mutations in synaptojanin through a powerful forward genetic screen. One, $synj^{1}$, is a nonsense mutation truncating the protein by about two-thirds, while the second, $synj^{2}$, is a missense mutation causing the substitution G65D. Both $synj^{1}$ and $synj^{2}$ are by genetic criteria strong loss-of-function mutations. Verstreken and colleagues show that loss of synaptojanin almost abolished stimulus-induced FM1-43 uptake at larval neuromuscular junctions (NMJs), indicating a defect in activity-dependent membrane uptake. Consistently, evoked transmitter release exhibited a rapid run-down during high-frequency stimulation, indicating a depletion of releasable vesicles. Ultrastructurally, synaptojanin mutant NMJs were largely depleted of synaptic vesicles, indicative of an endocytotic defect. Only a small number of vesicles remained in close approximation to the presynaptic membrane. Some of these were associated with active zones, presumably the remaining

pool of releasable vesicles. A second population was found away from active zones and contained intermediate structures of endocytosis. Often, regularly spaced coated vesicles were observed, indicative of an arrested cytoskeleton-vesicle interaction. However, synaptojanin mutant photoreceptor terminals were not severely depleted of vesicles but showed a significant accumulation of coated vesicles, which were regularly spaced in multiple parallel rows. The cause for the "weaker" defect in photoreceptor terminals is not known but could indicate a compensatory effect by a functionally related protein. Similar arrays of coated vesicles were found in mouse and *C. elegans* (Harris et al., 2000; Slepnev and De Camilli, 2000). Thus, genetic data from mice, flies, and worms consistently suggest that synaptojanin promotes multiple steps of clathrin-mediated endocytosis including cytoskeleton-vesicle interactions during endocytosis.

Erik Jorgensen's group (Schuske et al., 2003) analyzed two viable mutations in the *C. elegans* endophilin (unc-57). One, *ok310*, is a deletion truncating the protein by about half, while the second, *e406*, is a nonsense mutation causing a stop at position 130, truncating endophilin by about two-thirds. Both mutations *ok310* and *e406* affect the SH3 domain and the BAR domain of endophilin, the latter containing residues that are required for lipid binding and LPAAT activity. By genetic criteria, both mutations are at minimum severe loss-of-function mutations. Schuske and colleagues show that loss of endophilin caused a reduction of evoked release, which became more pronounced during higher-frequency stimulation (1 Hz), suggesting a depletion of releasable vesicles. Ultrastructurally, endophilin mutant synaptic terminals exhibited a depletion of clear vesicles and an accumulation of various endocytotic intermediates, including coated vesicles as well as shallow and deeply invaginated pits, which were typically found uncoated and adjacent to active zones. Moreover, some electron-dense vesicles appeared to be organized in long arrays connected through a filamentous network, giving the appearance of a "string-of-pearls" phenotype. Similar defects in synaptic vesicle recycling were found in *Drosophila* (Guichet et al., 2002; Verstreken et al., 2002). Thus, genetic data from flies and worms consistently suggest that endophilin is required for multiple steps of clathrin-mediated endocytosis including cytoskeleton-vesicle interactions.

The comprehensive phenotypic analysis of synaptojanin and endophilin mutants in *Drosophila* and *C. elegans* revealed a remarkable similarity of defects between mutations in endophilin and synaptojanin. Realizing the possible implication that both proteins may act in the same signaling pathway, both groups consequently went on to genetically test the significance of the implied endophilin-synaptojanin interaction in vivo. The genetic criteria are simple: if two proteins act in the same pathway, then the double mutant phenotype should be no more severe than the phenotype of individual mutants. Using sensitive assays, both groups show that the defects in endocytosis of endophilin-synaptojanin double mutants are qualitatively and quantitatively similar to the defects in the individual mutants. Together with the known biochemical interactions, these genetic data clearly establish that endophilin and synaptojanin act together in a common pathway.

Both groups further examined the localization of each protein in mutant animals and found a normal distribution of endophilin in synaptojanin mutants. However, protein levels of synaptojanin were much reduced in *Drosophila* and *C. elegans* endophilin mutants. Neither reduced protein transport nor reduced protein expression seemed likely to cause the reduced levels of synaptojanin. Accordingly, endophilin seems to stabilize synaptojanin at nerve terminals. Hence, this suggests that endophilin may recruit synaptojanin, possibly at an early step of endocytosis, and then shuttle synaptojanin through all subsequent stages. Such a function is consistent with the effects of overexpressing endophilin in synaptojanin mutant photoreceptors in *Drosophila*, which partially restored the defects caused by loss of synapojanin function. However, in *C. elegans*, neither overexpression of endophilin in synaptojanin mutants nor overexpression of synaptojanin in endophilin mutants had a significant effect. The cause for this difference between the two systems is not known. One explanation could be that synaptojanin's and endophilin's functions are so intricately interweaved that there is no separable upstream or downstream function if one of the protein activities is fully absent. Notably, the partial rescue of endocytosis by endophilin overexpression was observed in the less severely affected photoreceptors of the weaker *synj*^{2} allele of synaptojanin.

Although not a main point, still remarkable is the observation that neither the loss of endophilin nor the loss of synaptojanin completely blocked neurotransmitter release (Guichet et al., 2002; Verstreken et al., 2002). Interestingly, activity-dependent FM1-43 uptake at *Drosophila* NMJs of endophilin and synaptojanin mutants was severely reduced, if not abolished, indicating a block of endocytotic membrane uptake. But if so, why then is transmitter release not abolished during periods of high synaptic activity, like in *shibire* mutants affecting dynamin (Koenig et al., 1989)? Previous work indicates that FM1-43 only labels synaptic vesicle membranes undergoing clathrin-mediated endocytosis but not vesicle membranes undergoing "kiss-and-run" endocytosis (Klingauf et al., 1998). If this is correct, then mutations in endophilin and synaptojanin but not mutations in dynamin (*shibire*) uncover a kiss-and-run mechanism at fly NMJs. Consequently, this would suggest that dynamin but not endophilin or synaptojanin are required for kiss-and-run. Accordingly, dynamin should be present at hotspots of clathrin-mediated endocytosis and at active zones, where kiss-and-run is assumed to operate. However, so far dynamin has been localized only to hot spots of clathrin-mediated endocytosis but not to active zones (Estes et al., 1996). This may be a minor twist, but it is one that needs to be addressed.

In conclusion, both groups elegantly and convincingly demonstrate that the functions of endophilin and synaptojanin during endocytosis are tightly linked and pleiotropic. The stabilization of synaptojanin through its interaction with endophilin supports the view for an adaptor role of endophilin. However, adaptor might not be the proper term. The endophilin/synaptojanin interaction is remarkable in that it combines two different enzymatic activities in one "paired enzyme complex" that seems to stay with a newly forming vesicle for most of its endocytotic passage. As indicated by a multitude of defects,

PI(4,5)P2 degradation by synaptojanin and/or a lipid-shaping function by endophilin might be sequentially and/or cooperatively required for the transition of shallow into deeply invaginated pits, the fission of vesicles from the donor membrane, the uncoating of vesicles, and for cytoskeletal interactions of endocytosing vesicles. Hence, it will be important, though difficult, to dissect when and where each individual enzymatic activity is required during endocytosis. As usual, obtaining some answers leads only to more questions.

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Glycine Transporters Not Only Take Out the Garbage, They Recycle

Two articles in the current issue of *Neuron* examine the consequences of deleting the two genes that encode glycine transporters. Interestingly, loss of glial transporters enhances while loss of presynaptic neuronal transporters reduces glycinergic transmission. These two opposing phenotypes resemble distinct human diseases characterized by dysfunction in glycinergic signaling.

After release, most neurotransmitters are removed from the vicinity of the synapse by specialized plasma membrane transporters. Like tiny garbage men, these integral membrane proteins perform a thankless task, clearing the refuse of neurotransmission from the synapse. In the absence of such cleanup, circuits become littered with excess neurotransmitter. Allowing neurotransmitters to pile up not only impedes signaling, it drains resources by permitting a valuable, reusable commodity to go to waste. In the delicate economy of the synapse, then, it is up to these garbage men to regulate the fine balance between recycling and consumption.

In order to meet these needs, multiple genes encode transporters for certain neurotransmitters, such as glutamate (five genes), GABA (four genes), and glycine (two genes). For the two glycine transporters GlyT1 and GlyT2, unique expression patterns suggest specialized functions. The neuronal transporter GlyT2 is largely confined to presynaptic terminals that secrete glycine, and so its localization matches the distribution of glycinergic neurons found mainly in the brain stem and spinal cord (Jursky and Nelson, 1995; Zafra et al., 1995). Because of this overlap, a prevailing view has been that GlyT2 is the principal pathway for reuptake of glycine released at glycinergic synapses. In comparison, a much broader expression pattern is observed for the glial isoform GlyT1 (Zafra et al., 1995). This transporter is located in regions of the brain not known to rely on glycinergic inhibition (cortex, hippocampus, thalamus), suggesting that it has another role. Since glycine is also known to act as a coagonist of excitatory NMDA receptors, it has been hypothesized that GlyT1 influences excitatory synaptic signaling by regulating ambient glycine concentrations (Smith et al., 1992; Berger et al., 1998; Chen et al., 2003). Thus, although the reasons for genetic redundancy are uncertain, it is appealing to think that individual transporters have specialized functions.

In this issue of *Neuron*, papers by Gomeza and Hülsmann et al. (Gomeza et al., 2003a) and Gomeza et al. (2003b) test these assumptions by generating two lines of knockout mice, one deficient in the glial isoform and the other lacking the neuronal isoform of glycine transporter. Avoiding the pitfalls of pharmacological manipulation, these studies provide the clearest view yet of specific roles played by glial and neuronal glycine uptake.

In the first of the two papers, Gomeza and Hülsmann et al. inactivate the glial transporter gene. Genotyping these homozygous mutant mice (*GlyT1*$^{-/-}$) confirmed that they were devoid of the wild-type allele. Further analysis showed that, while both GlyT1 transcript and protein were absent in the mutants, GlyT2 remained unaffected. As expected from the normal distribution of GlyT1, tissue originating from both forebrain and brain stem regions of *GlyT1*$^{-/-}$ mice exhibited significantly reduced uptake of radiolabeled glycine.